PROC. ENTOMOL. SOC. WASH.
99(3), 1997, pp. 513–522

COMPARISON OF FIXATION AND DRYING PROCEDURES FOR SCANNING ELECTRON MICROSCOPY AMONG INSECT BODY TYPES

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Abstract.—Four different procedures used to prepare insect specimens for scanning electron microscopy were compared using three insect body types that represent unique obstacles to obtaining good quality micrographs, i.e., clean and life-like in appearance. The micrographs produced using each procedure were rated for cleanliness, structural integrity, and imaging quality. Each procedure involved: fixation, post-fixation, and drying. Procedure 1 involved fixation by freezing @ −10°C and drying by critical-point drying. Procedure 2 involved fixation by submersion in 70% ethanol and drying by critical-point drying. Procedure 3 involved fixation by submersion in 70% ethanol and drying with hexamethyldisilizane. Procedure 4 involved fixation by submersion in a hot water bath at 80°C and drying with hexamethyldisilizane. Procedure 2 gave the best overall results, but procedures 3 and 4 were best on insects with small lightly sclerotized bodies and unsclerotized larvae, respectively. Difficulties associated with histological study of insects are discussed.

Key Words: Scanning electron microscopy, freezing fixation, ethanol fixation, hot water fixation, procedures

Scanning electron microscopy (SEM) is used for a variety of purposes in Entomology. The most common applications involve taxonomic and morphological studies. While SEM is often an indispensable tool in these types of studies, the quality of the images can vary considerably; much of this variation is related to problems specific to insects. Quality images useful for scientific study require that the specimens be clean and life-like in appearance (i.e. little or no shrinkage).

The procedures for preparation of insect specimens for SEM are fairly standard, although variations during fixation and dehydration are common (see below). Papers that include SEM micrographs may not describe their procedures or simply acknowledge an individual or department for their help in the production of SEM micrographs, thus, information on new or different successful techniques are difficult to obtain. Insects present a wide range of histological variables, and no one treatment is universally applicable to every taxa or stage. The objective of this study was to compare different procedures used on different insect body types to determine which, if any, provided consistent and predictable results, or which procedure was best suited with selected taxa to obtain clean, life-like specimens.

For the purposes of this paper, we assume the reader has basic knowledge of SEM preparation methods; however, the following terms are defined:

Accelerating voltage.—Accelerating voltages are typically set at ca. 10–20kV for biological tissues. The resultant resolution of surface detail usually does not exceed 10,000 diameters of magnification.

Fixation.—The cessation of life in such a manner as to retain the structural and biochemical integrity of the specimen and its tissues. See Sabatini et al. (1963), Humanson (1967), Barbosa (1974), and Dawes (1988) for basic principles of fixation. This is the most important step in histological preparation.

Postfixation.—There are many postfixation processes for biological tissues depending on the examination method. Metallic impregnation with heavy metal salts is commonly used for SEM (Sabatini, et al. 1963, Dawes 1988). The most common metallic salt is osmium tetroxide (OsO_4) which acts to bind lipids, thus increasing the electron density of the tissues. Electron density in biological tissues allows for higher accelerating voltages without charging, with the subsequent gain in resolution. Enhanced contrast of the specimen also can result from metal impregnation leading to a better quality image for study (Dawes 1988). OsO_4 is labeled as a poison. A Material Safety Data Sheet is provided with the purchase of OsO_4; precautions on proper handling and disposal should be followed.

Drying (Dehydration).—Tissues must be completely dry, including absence of metabolic water, before sputter-coating and examination with SEM. There are several drying procedures. The most common is the use of a critical-point dryer with liquid CO_2 as the transition fluid (Dawes 1988, Gordh and Hall 1979). Hexamethyldisilizane (HMDS) is another chemical which dries tissues (Adams et al. 1987, Nation 1983) and is employed in some of the procedures listed below. HMDS is labeled as corrosive and highly toxic. Again, a Material Safety Data sheet is provided with purchase and procedures for proper handling should be followed.

Body types.—Three distinct body types, difficult to successfully prepare for SEM, were selected for analysis A) small bodies (<2 mm long), B) bodies that produce wax and C) soft-bodied forms, which include both immature stages or insect imagos and immatures with little sclerotization of the integument. These types are referred to as 'small', 'waxy' or 'soft' body types. We used *Encarsia* sp. (Hymenoptera: Aphelinidae) a parasitoid that is ca. 1.0 mm long, to represent the small body type. For waxy body types, we used the nymphs of two species of whitefly, *Bemisia argentifolii* Bellows and Perring and *Dialeurodes citri* (Ashmead) (Homoptera: Aleyrodidae). The nymphs of these two whitefly species also are soft-bodied. We used late-instar larvae of *Musca domestica* L. (Diptera: Muscidae) to represent the soft body type.

Specimen Preparation

The problems associated with insects and their preparation for SEM involve primarily the cuticle, and secondarily, the presence of waxes and lipids. All insects possess a multi-layered cuticle which presents a unique obstacle to histology and, depending on the taxa involved, may be heavily sclerotized or rather thin and flexible (Humason 1967, Barbosa 1974).

The cuticle provides a barrier to the materials used in each step in specimen preparation: fixation, postfixation and drying (Barbosa 1974, Dawes 1988). During fixation, the cuticle can prevent adequate penetration of the fixative into the body, and thorough fixation of tissues. Poor fixation may also cause problems later during postfixation as tissues that have not been fixed will not accept the post-fixative, and may be more susceptable to electron absorption which results in charging (Dawes 1988). Drying of the tissues may also be hampered by partial fixation, allowing water and alcohol to remain in the specimen. Such tissues may burst when placed in a vacuum during sputter-coating and SEM examination. Metamorphosis also poses special problems during processing of tissues for SEM, for if the specimen being processed is in a period of structural rearrangement, the cuticle may be prone to shrinkage and severe charging problems.

Other problems associated with SEM ex-

amination of insects involve the presence of waxes and lipids. All biological tissues contain lipids, which tend to absorb electrons, and thus lead to charging. Many insects also produce waxes which also do not fix well, absorb electrons, and may obscure certain structures. The post-fixative, osmium tetroxide, is typically used to bind lipids to make them more electron dense, thus eliminating electron absorption and consequent charging (Dawes 1988).

SEM facilitates examination of the surface features of very tiny structures. Many insects are quite small or bear minute structures on their bodies. Although easily penetrated by preparation materials, these small-bodied insects pose difficulties during examination as they tend to become electrically charged.

With these problems in mind, we applied four procedures to specimens of taxa which represent the three body types described above. Comparisons of the procedures are presented with a rating scale for each.

Materials and Methods

Each procedure involved three basic steps: 1) fixation, 2) post-fixation, and 3) drying. Three methods of fixation were employed in the current study: 1) freezing @ −10°C, 2) submersion in ethanol (50–70%), and 3) a hot water bath at 80°C. A 2% aqueous solution of osmium tetroxide was always used as the postfixative. Metallic impregnation as a postfixative may not be commonly used for some insect taxa, but has been shown to be beneficial and thus is included in each procedure (Dawes 1988, Headrick, unpublished data). Dehydration involved placing the specimens in a series of ethanol baths at dilutions of 15, 30, 50, 70, 80, 90, 95 and finally 100%, followed either by critical-point drying using liquid CO_2 as a transition fluid, or HMDS. Following the 100% ethanol bath in two procedures the specimens were placed in HMDS under a fumehood. After 20 min. the remaining HMDS was siphoned away with a pipette, thus removing any accumulated particles or residues. A second bath of clean HMDS was added and the specimens were dried to completion by evaporation of the HMDS in a fumehood (ca. 30 min).

Procedure 1.—This method used freezing @ −10°C for fixation. The specimens were postfixed by immersion in the solution of osmium tetroxide for 24 h. They were next rinsed twice with deionized water, then dehydrated through a series of increasing ethanol dilutions. Specimens remained in each dilution for 20 min and ended in 100% ethanol. Drying used critical-point drying. This procedure was applied to 10 specimens each of *Dialeurodes citri, Bemisia argentifolii,* and *Encarsia* sp.

Procedure 2.—Specimens were fixed in 50% ethanol for 24 h, then rehydrated to distilled water through a decreasing series of ethanol dilutions for post-fixation in osmium tetroxide. Postfixation, dehydration, and drying were identical to procedure 1. Procedure 2 was applied to 10 specimens each of *D. citri, B. argentifolii,* and *E.* sp.

Procedure 3.—Fixation was in 70% ethanol, and postfixation in 2% osmium tetroxide for 24 h. The specimens were then dehydrated through a series of increasing ethanol dilutions up to 100% for 15 min in each dilution. The specimens were dried using two, 30-min baths of HMDS. This procedure was applied to 10 specimens of *E.* sp.

Procedure 4.—Specimens were fixed in a bath of 80°C tap water and subjected to ultrasonic cleaning for 10 min to remove residue from the body (Belcari 1987). After 5 min of fixation, the specimens were cut in half to facilitate the fixation process. The specimens were then post-fixed in osmium tetroxide for 24 h, and rinsed twice with deionized water. The specimens next were dehydrated in an increasing series of ethanol dilutions, up to 100%; each ethanol bath lasted 20 min. Two 30 min baths in HMDS finally were used to dry the specimens. This procedure was applied to 12 specimens of *M. domestica.*

All dried specimens were mounted on

aluminum stubs with double-sided cellophane tape and sputter-coated with a gold-palladium alloy using a Hummer V. Specimens were examined on a JEOL JSM C35 scanning electron microscope in the Department of Nematology at the University of California, Riverside. Scanning electron micrographs were prepared at 15 kV accelerating voltage on Polaroid 55P/N film.

To quantify the results of our comparative survey, we used the following rating system. We judged SEM micrograph quality on three variables: cleanliness, structural integrity, and imaging. Each of these categories had two subdivisions. We rated cleanliness at two levels, "particle" and "obscuring." Particle is macroscopic accumulation of atmospheric or environmental debris by the specimen. Particle accumulation can take place during any phase of the processes used for specimen preparation. Obscuring is a microscopic phenomenon in which materials such as waxes, resins or other natural products are chemically affected by the SEM preparation processes and adhere to the surface of the specimen, thus obscuring minute surface details. Similar obscuring problems arise from metallic filming or sputter-coating processes (Dawes 1988). The rating system ranged from 0–3 in both categories. For particle cleanliness, a 0 indicated that the specimen was covered with particles, and thus rendered useless for study (Fig. 1A); while 3 meant the specimen was free of any obscuring particles. With respect to obscuring residues, a 0 meant minute structures were obscured from view, and a 3 indicated that all structures could be seen clearly.

Structural integrity includes both macroscopic and microscopic levels. Macroscopic structural integrity encompasses the body as a whole. Microscopic structural integrity relates to minute structures on the body, such as setae or sensoria. We rated both of these categories as good, fair or poor. Good structural integrity evidenced little to no structural collapse. Fair structural integrity may have some collapse, but not loss of useable micrographs for study. Poor structural integrity resulted from collapse of the specimen, or loss of small structural details, preventing observation of important features (Fig. 1B, C).

Imaging includes resolution and charging. Resolution at magnifications greater than 2000× was rated as 0–3. A 0 indicated that the image could not be adequately resolved and a 3 indicated that resolution above 10,000× was obtainable. Charging in specimens is either present (+) or absent (−) (Fig. 1D).

Our rating system is summarized as follows and was used to build Tables 1–3:

A. Specimen cleanliness:
   1. Particle—0–3
   2. Obscuring—0–3
B. Structural Integrity:
   1. Macroscopic—Good, fair, or poor
   2. Microscopic—Good, fair, or poor
C. Imaging:
   1. Resolution at higher magnifications (>2000): 0–3
   2. Charging: present (+) or absent (−).

Results

Procedure 1.—Freezing fixation

Dialeurodes citri.—With this procedure specimens were generally clean and no structures were obscured from view (Fig. 2A). At higher magnification, resolution was rated as 1 (Fig. 2B). Collapse of the body and its smaller structures was not observed and charging did not occur with these specimens.

Bemisia argentifolii.—Cleanliness with respect to particles was good (Fig. 2C), but most minute structures were obscured by waxes. Because the specimens were not clean, the tracheal furrows were left full of wax (Fig. 2D); this was more life-like, but obscured potentially important taxonomic characters. Severe charging was present in some specimens. Resolution at higher magnification was rated as 1.

Encarsia sp.—The specimens bore obscuring residues (Fig. 2E). Macroscopic and

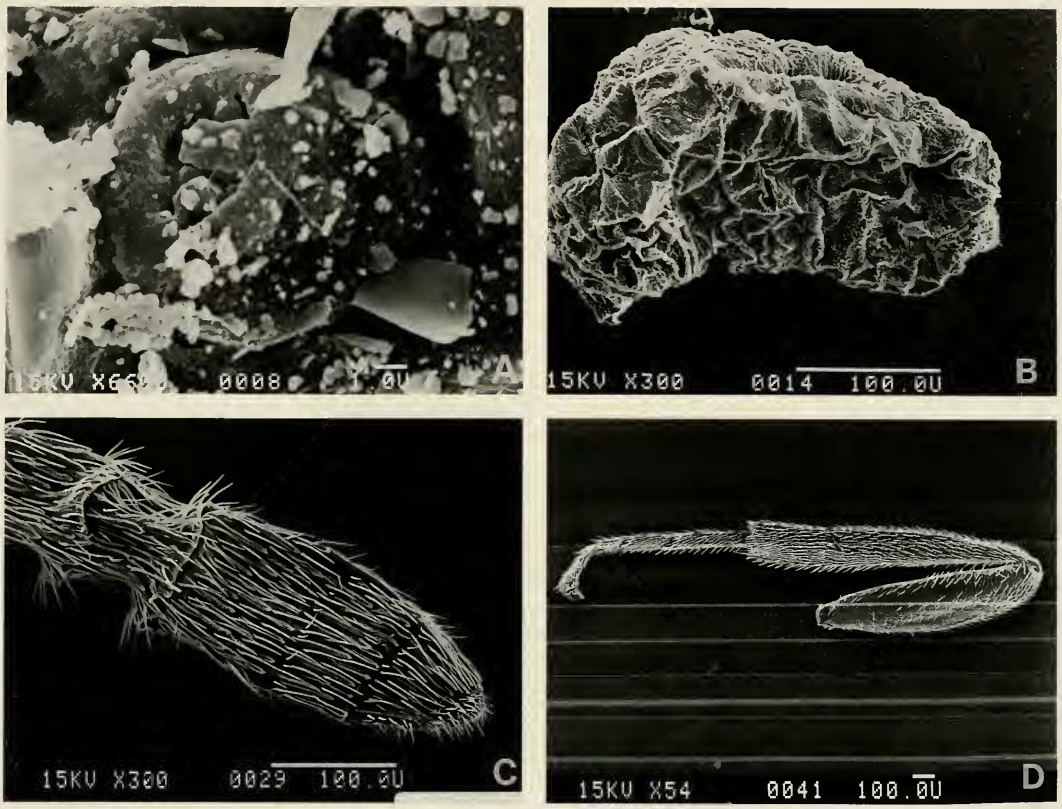

Fig. 1. Examples of poor SEM micrographs. A, Surface details obscured by particulate matter, this should look like Figure 5C. B, Shinkage of soft-bodied dipteran larva. The shrinkage on this specimen was so severe that even the most minute of structures was collapsed. C, Shinkage of a hymenopteran parasitoid antenna. D, An example of what charging does to a micrograph.

Table 1. Rating results for Procedure 1, freezing fixation.

Rating	Taxa		
	D. citri	*B. argentifolii*	*E.* sp.
Cleanliness			
Particle	2	2	2
Obscuring	2	1	1
Structural integrity			
Macro	good	good	poor
Micro	good	fair	poor
Imaging			
Resolution	1	1	0
Charging	(−)	(+)	(−)

Table 2. Rating results for Procedure 2, ethanol fixation.

Rating	Taxa		
	D. citri	*B. argentifolii*	*E.* sp.
Cleanliness			
Particle	2	2	2
Obscuring	2	2	2
Structural integrity			
Macro	good	good	good
Micro	good	good	good
Imaging			
Resolution	2	2	1
Charging	(−)	(−)	(−)

Table 3. Rating results for Procedures 3, ethanol fixation and HMDS, and Procedure 4, water bath fixation and HMDS.

Rating	Procedure 3 *E.* sp.	Procedure 4 *M. domestica*
Cleanliness		
Particle	2	3
Obscuring	3	3
Structural integrity		
Macro	fair	good
Micro	good	good
Imaging		
Resolution	3	3
Charging	(−)	(−)

microscopic structures showed considerable collapse (Fig. 2F). No charging occurred, but resolution at higher magnifications was rated as 0.

Procedure 2.—Ethanol fixation

Dialeurodes citri.—The specimens were free of particles and obscuring residues (Fig. 3A). Resolution at higher magnification was rated as 2 (Fig. 3B). Collapse and charging were not problems.

Bemisia argentifolii.—The specimens (Fig. 3C), and specifically, the tracheal furrows were free of waxes (Fig. 3D). Resolution was rated as 2 at higher magnification. There was no structural collapse or charging.

Encarsia sp.—The specimens were clean with little collapse (Fig. 3E). At higher magnifications, the resolution obtained was rated as 1 (Fig. 3F).

Procedure 3.—Ethanol fixation, HMDS

Encarsia sp.—The specimens were mostly free of particles or obscuring residues (Fig. 4A). Microscopic collapse of structures was not a problem with this procedure (Fig. 4B, C); although there was some macroscopic collapse in the area of the propodeum (Fig. 4A). Resolution was extremely good up to 18,000× (Fig. 4C), and there was no charging.

Procedure 4.—Hot water fixation/ sonication, HMDS

Musca domestica.—The specimens were very clean with minimal obscuring residues (Fig. 5A, B, C). No collapse was evidenced and the smallest structures remained lifelike in appearance (Fig. 5C). Resolution was excellent and there was no charging.

Discussion

Casual examination of entomological journals reveals a considerable range in the quality of SEM micrographs. Technological advances in SEM's have made them easier to use and have provided the user with more options for producing good quality micrographs. Adjusting the physical parameters of the SEM, such as, accelerating voltage, stigmator, focus, and spot size can now be done with the touch of a button; adjusting features of the image like contrast and brightness is also made simpler. Most of the newer SEM's have the ability to digitize images and save them as files for importation into image-enhancing software. Once stored as a digitized image the user can then opt for "cleaning-up" or enhancing image quality after the fact.

Although technology is moving ahead on the hardware end, the fact remains that preparation of the specimen is still the most important first step and this is the area where little comparative work on procedures has been conducted. Some new techniques reported in the literature involve lengthy processing, the use of chemicals or materials that are difficult to handle, or specialized equipment not commonly available (Grodowitz et al. 1982; Colwell and Kokko 1985). While these expenditures of time and resources may be useful for intensive study of a paticular taxon, the applicability of these methods to other insect taxa or to specimens of different body types is limited.

Our comparative analysis of methods commonly used for SEM preparation among different types of insects is a first

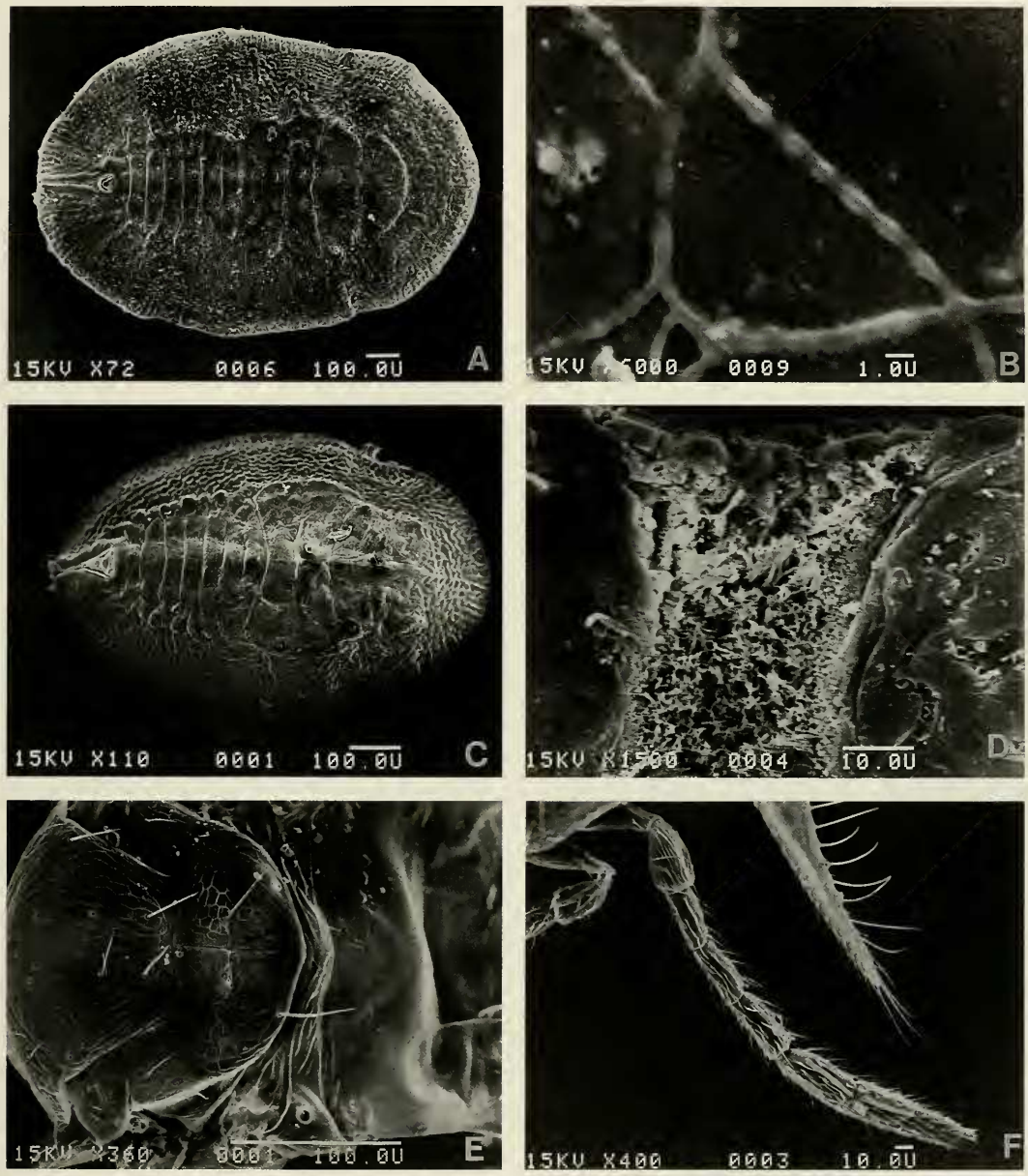

Fig. 2. Freezing fixation. A, *Dialeurodes citri* fourth instar nymph, dorsal view. B, *D. citri,* detail of dorsal integumental reticulation. C, *Bemisia argentifolii* fourth instar nymph, dorsal view. D, *B. argentifolii,* detail of the ventral marginal opening of the tracheal furrow. E, *Encarsia* sp., female, dorsal view of the thoracic region. F, *E.* sp., female, antenna.

step in determining the applicability of these methods in obtaining consistent, quality specimens.

The procedure which ranked the highest among all four taxa tested was ethanol fixation, followed by osmium tetroxide post-fixation, dehydration in ethanol, and critical-point drying. This procedure provided generally clean specimens with little collapse or charging. Its limitation may be the loss of secreted surface features, such as wax, thus, rendering the specimen "unlife-

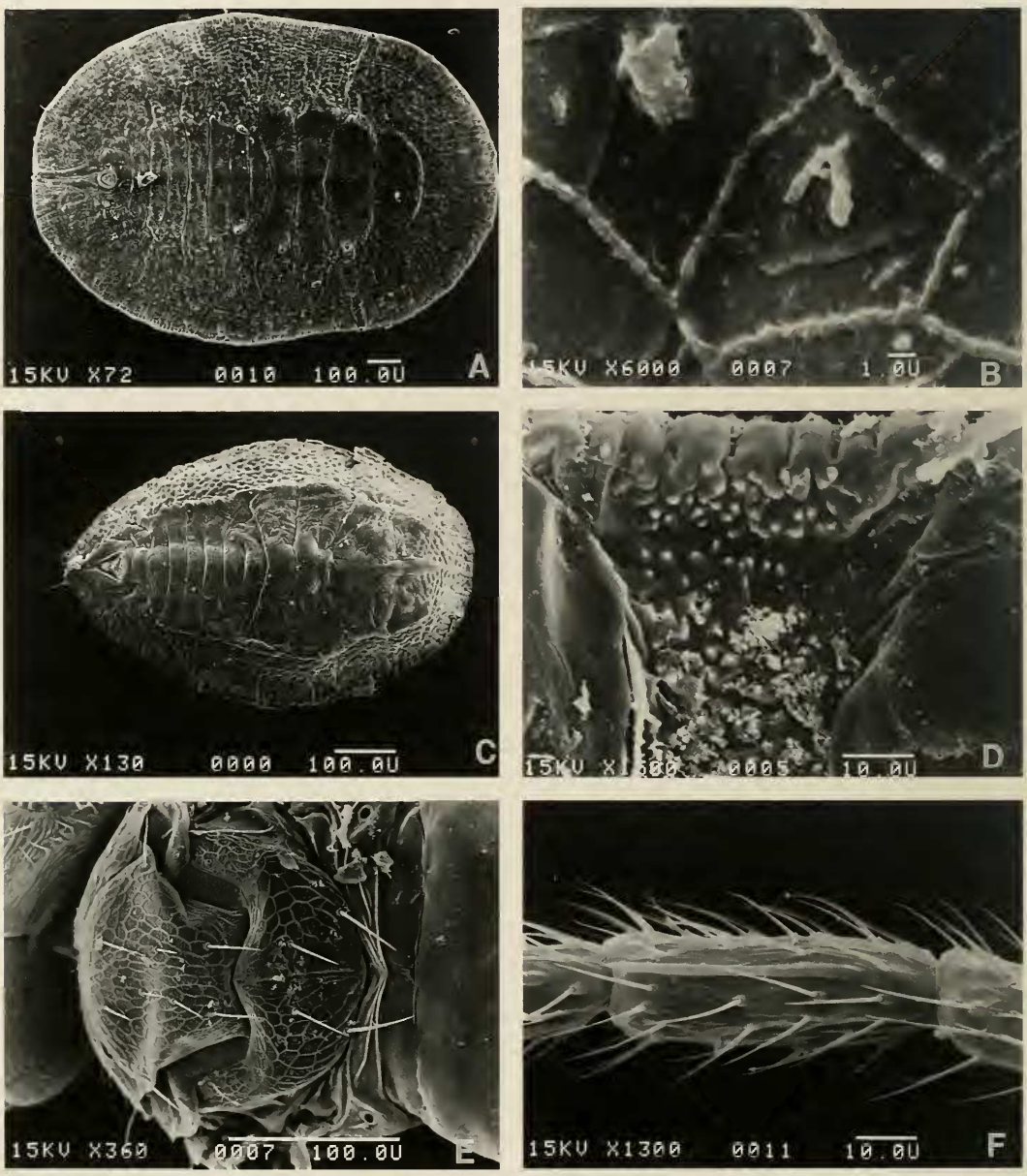

Fig. 3. Ethanol fixation. A, *Dialeurodes citri* fourth instar nymph, dorsal view. B, *D. citri,* detail of dorsal integumental reticulation. C, *Bemisia argentifolii* fourth instar nymph, dorsal view. D, *B. argentifolii,* detail of the ventral marginal opening of the tracheal furrow. E, *Encarsia* sp., female, dorsal view of the thoracic region. F, *E.* sp., female, detail of a funicle segment of the antenna.

like", but again, better suited to viewing integumental surface features. This method has also been used successfully to examine a wide array of insect taxa and insect stages (DHH, unpublished data). Ethanol fixation has produced the best and most consistent results for SEM examination of insect eggs (DHH, unpublished data). In the present study, fixation by freezing also gave good results and eliminated the need for the extra rehydration steps before postfixing, as is needed with ethanol fixation. Procedures 3

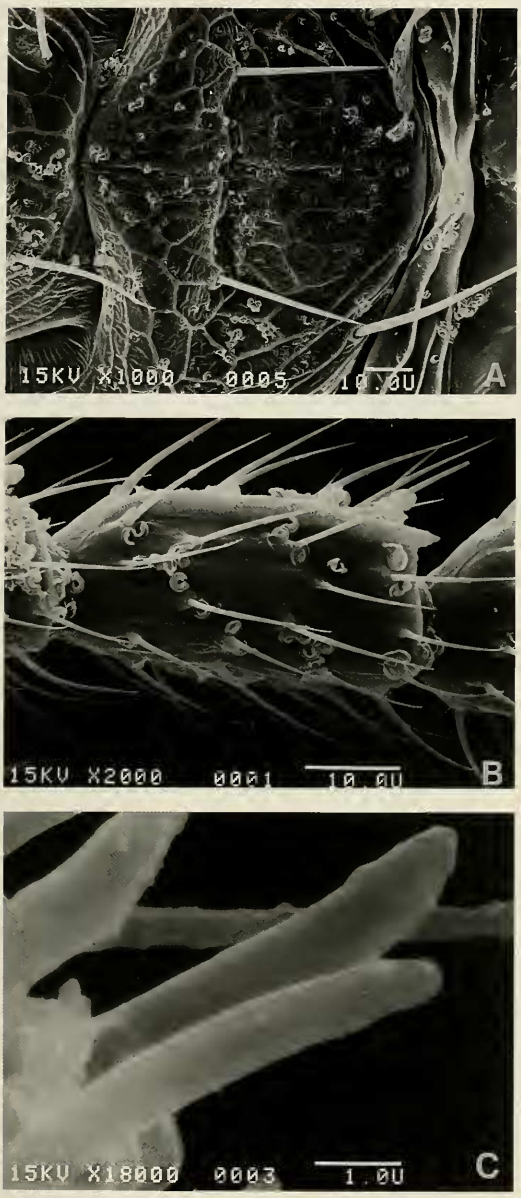

Fig. 4. Ethanol fixation, HMDS, *Encarsia* sp., A, Female, dorsal view of the thoracic region. B, Female, detail of a funicle segment of the antenna. C, The apex of the antennal club.

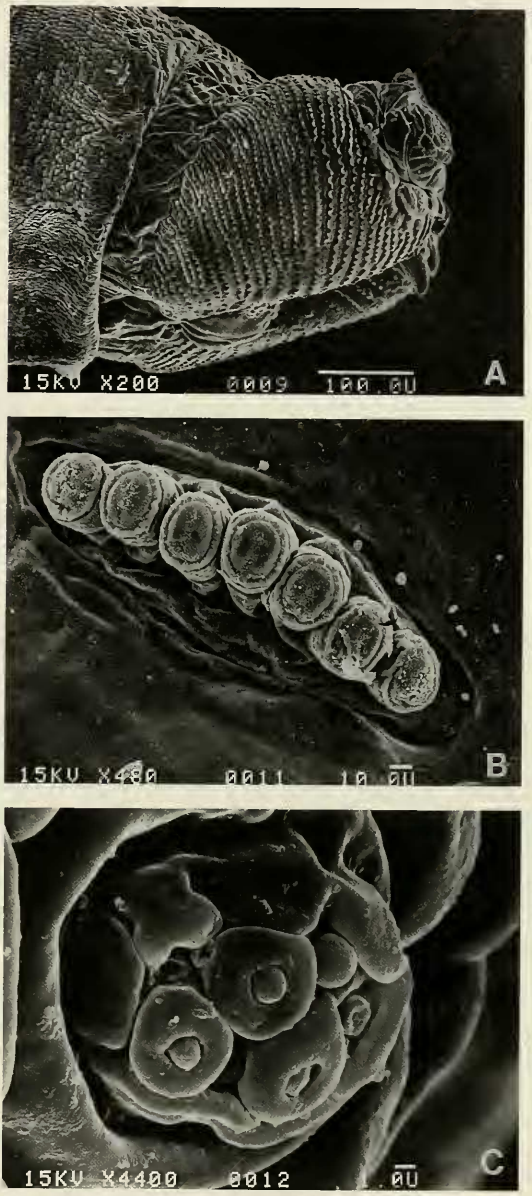

Fig. 5. Hot-water fixation, HMDS, *Musca domestica.* A, Gnathocephalon, right lateral view. B, Anterior prothoracic spiracles. C, Terminal sensory organ of the anterior sensory lobe.

and 4 gave excellent results, but were not widely applicable. Dipteran larvae are unusually prone to shrinkage and distortion during processing for SEM. Further, they are often contaminated with debris and other by-products of their various micro-habitats that are not easily removed by chemical means, e.g., baths in hexanes, bleach, etc. Other taxa such as lepidopteran or coleopteran larvae can withstand such harsh treatments without damage. We have found that procedure 4 works exceptionally well to

provide clean, undistorted larvae; larger-bodied forms are cut apart to facillitate penetration during the fixation process.

The procedure that may be considered the best will vary with the ultimate goal of the user. For example, life-like appearance may not be compatible with ethanol fixation. More taxa and body types must be studied in a comparative fashion. The inclusion of preparation methods in the literature, however briefly, would aid development of procedures that predictably provide clean, life-like specimens and good quality SEM images.

Acknowledgments

We thank Carol Meisenbacher and Bobbie Orr for technical assistance and the use of specimens from their colonies. We also thank R. D. Goeden and J. M. Heraty for reviews of early drafts of this manuscript.

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